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May atyid shrimps act as potential vectors of crayfish plague?

Agata Mrugała[1], Miloš Buřič[2], Adam Petrusek[1], Antonín Kouba[2]

1 *Department of Ecology, Faculty of Science, Charles University, Viničná 7, Prague 2 CZ-12844, Czech Republic* **2** *University of South Bohemia in České Budějovice, Faculty of Fishery and Protection of Waters, South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic*

Corresponding author: *Agata Mrugała* (agata_mrugala@wp.pl)

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Abstract

The causative agent of crayfish plague, *Aphanomyces astaci* Schikora, was long considered to be a specialist pathogen whose host range is limited to freshwater crayfish. Recent studies, however, provided evidence that this parasite does not only grow within the tissues of freshwater-inhabiting crabs but can also be successfully transmitted by them to European crayfish species. The potential to act as alternative *A. astaci* hosts was also indicated for freshwater shrimps. We experimentally tested resistance of two freshwater atyid shrimps: *Atyopsis moluccensis* (De Haan, 1849) and *Atya gabonensis* Giebel, 1875. They were infected with the *A. astaci* strain associated with the globally widespread North American red swamp crayfish, *Procambarus clarkii* (Girard, 1852), the typical host of the *A. astaci* genotype group D. As popular ornamental species, both shrimps may get in contact with infected *P. clarkii* not only in the wild but also in the aquarium trade. We assessed the potential of shrimps to transmit *A. astaci* to susceptible crayfish by cohabiting *A. gabonensis* previously exposed to *A. astaci* zoospores with the European noble crayfish, *Astacus astacus* (Linnaeus, 1758). In both experiments, the presence of *A. astaci* infection was analysed with species-specific quantitative PCR. We detected *A. astaci* in bodies and exuviae of both shrimp species exposed to *A. astaci* zoospores, however, the intensity of infection differed between the species and analysed samples; it was higher in *A. moluccensis* and the exuviae of both species. *A. astaci* was also detected in one *A. astacus* individual in the transmission experiment. This finding reveals that freshwater shrimps may be able to transmit *A. astaci* to crayfish hosts; this is particularly important as even a single successful infection contributes to the spread of the disease. Moreover, our results indicate that the tested shrimp species may be capable of resisting *A. astaci* infection and reducing its intensity through moulting. Although their potential to act as prominent *A. astaci* vectors requires further research, it should not be ignored as these freshwater animals may then facilitate *A. astaci* spread to susceptible crayfish species in aquarium and aquaculture facilities as well as in the wild.

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Keywords
Aphanomyces astaci, aquatic invasion, *Cherax destructor*, disease transmission, oomycetes, species introduction

Introduction

Invasive alien species (IAS) are considered one of the major threats to native biodiversity (Sala et al. 2000), due to their wide range of negative impacts on the functioning of whole ecosystems and their communities (Blackburn et al. 2014). Moreover, IAS represent a significant source of non-native pathogens whose transmission to susceptible hosts may have unforeseeable consequences (Roy et al. 2017). The IAS may not only be responsible for an introduction of novel disease agents but also facilitate the spread of the ones that already occur in their new ranges (Peeler et al. 2011; Strauss et al. 2012). In fact, one quarter of the IAS listed as the 100 of the "world's worst" (Lowe et al. 2004) cause environmental impacts linked to disease emergence, as disease agents, vectors or reservoirs (Hatcher et al. 2012).

The emergence in Europe of the oomycete *Aphanomyces astaci* Schikora, the causative agent of crayfish plague, exemplifies the devastating impacts that a novel pathogen may impose on native fauna. Its spread across the continent caused irreversible declines of native European crayfish populations and still threatens their remaining stocks (Alderman 1996; Holdich et al. 2009), leading to its inclusion among the worst IAS in Europe (Vilà et al. 2010) as well as worldwide (Lowe et al. 2004).

In Europe, the spread of *A. astaci* is mainly facilitated by its original hosts, North American crayfish species (Holdich et al. 2009; Rezinciuc et al. 2015). Thanks to their long co-evolutionary history with this pathogen, North American crayfish species are able to efficiently limit pathogen growth, and thereby act as asymptomatic carriers. In contrast, European native crayfish, and presumably all other crayfish species that do not originate from North America, are considerably more susceptible to *A. astaci* (reviewed in Svoboda et al. 2017). This is reflected, for instance, in the mass mortalities of endemic Japanese crayfish *Cambaroides japonicus* (De Haan, 1841) in Hokkaido, Japan (Martín-Torrijos et al. 2018) as well as of the farmed Australian redclaw *Cherax quadricarinatus* (von Martens, 1868) in Taiwan (Hsieh et al. 2016), both caused by *A. astaci*. Like in Europe, *C. japonicus* mortalities in Japan highlight that the spread of North American crayfish species on other continents may be followed by crayfish plague outbreaks with serious negative impacts (Mrugała et al. 2017). Therefore, this crayfish pathogen should be considered as a serious threat to susceptible indigenous crayfish populations around the world.

The releases and escapes from aquaculture and aquarium trade were assessed as the most important entry pathways of non-native freshwater species in Europe (Nunes et al. 2015). Likewise, the first introductions of North American crayfish into European freshwaters are associated with stocking to open waters and aquaculture (Holdich et al. 2009), and in recent years with illegal stocking activities, bait introductions, garden pond escapes and aquarium releases (Chucholl 2015; Patoka et al. 2017 and references therein). Indeed, the trade in ornamental crayfish species is nowadays considered as the main introduction pathway of non-indigenous crayfish species into European

freshwaters (Chucholl 2015; Kotovska et al. 2016; Weiperth et al. 2017, 2019a; Hossain et al. 2018). Moreover, *A. astaci*-infected ornamental crayfish species have been already reported in German, Czech, and even Indonesian aquarium trade (Mrugała et al. 2015; Panteleit et al. 2017; Putra et al. 2018), and hence releases of infected crayfish may further contribute to crayfish plague spread.

A. astaci was long considered to be a specialist pathogen whose host range is limited to freshwater crayfish (Decapoda: Astacoidea and Parastacoidea). Recent studies, however, confirmed assumptions of Benisch (1940) and Unestam (1972) about the carrier status of freshwater-inhabiting crabs (Decapoda: Brachyura). The Chinese mitten crab *Eriocheir sinensis* H. Milne-Edwards, 1853, *Potamon potamios* (Olivier, 1804) and *Parathelphusa convexa* de Man, 1879 were observed to carry *A. astaci* infection that they likely acquired from coexisting crayfish populations (Schrimpf et al. 2014; Svoboda et al. 2014a; Tilmans et al. 2014; Putra et al. 2018). Schrimpf et al. (2014) also demonstrated that *A. astaci* could be transmitted from infected *E. sinensis* to susceptible noble crayfish, *Astacus astacus* (Linnaeus, 1758). Moreover, the resistance to *A. astaci* was also tested in two freshwater shrimp species (Decapoda: Caridea): *Macrobrachium dayanum* (Henderson, 1983) and *Neocaridina denticulata davidi* (Bouvier, 1904) (Svoboda et al. 2014b). The experimental infection did not cause mortality in either shrimp species; however, their apparent resistance to the pathogen has been attributed to the purgatory effect of their frequent moulting. The results also indicated that some growth of *A. astaci* might have occurred in non-moulting individuals of *M. dayanum* and their exuviae, highlighting the potential of at least some shrimp species to act as *A. astaci* temporary hosts. This assumption was further supported by the detection of *A. astaci* in freshwater shrimp *Macrobrachium lanchesteri* (de Man, 1911) coexisting with infected red swamp crayfish *P. clarkii* (Girard, 1852) in Indonesia (Putra et al. 2018). However, no infection was detected in marine and brackish water crabs and shrimps in the Black Sea basin despite their proximity to infected populations of *Pontastacus leptodactylus* (Eschscholtz, 1823), supporting the assumption that the distribution and dispersal of *A. astaci* is restricted to freshwaters (Panteleit et al. 2018).

Apart from their ecological significance, many freshwater shrimps and crabs are involved in intensive aquaculture and pet trade, and hence they have considerable socioeconomic importance. Their potential sensitivity towards the crayfish plague pathogen might thus have far-reaching consequences (Svoboda et al. 2014b). Moreover, even if *A. astaci* infection is not accompanied with mortality in shrimps, they may still serve, similarly to North American crayfish in Europe, as chronic carriers of the pathogen, representing threats to wild populations and farms culturing susceptible crayfish species. Indeed, recent reports attribute the presence of ornamental shrimp species in European freshwaters to releases by hobbyists who keep them as aquarium pets (e.g., Klotz et al. 2013; Jabłońska et al. 2018; Weiperth et al. 2019b). The lack of reported mass-mortalities of *E. sinensis* in Europe, where it coexists in many rivers with North American crayfish, permits the assumption that at least this crab species is resistant to *A. astaci* infection (Schrimpf et al. 2014). Nevertheless, the situation is less clear for freshwater shrimp species.

The present study focuses on interactions of freshwater shrimp species with *A. astaci*, and experimentally tests two hypotheses evaluating shrimps' potential to act as its alter-

native vectors: 1) the chosen shrimp species may host *A. astaci*, and 2) they may transmit this parasite to susceptible crayfish. Two widespread filter-feeding atyid shrimps (Decapoda: Caridea) frequently traded for ornamental purposes were chosen: *Atya gabonensis* Giebel, 1875 originating from West Africa, and *Atyopsis moluccensis* (De Haan, 1849) from South-East Asia (Hobbs and Hart 1982; Chace 1983; De Grave and Mantelatto 2013). We may presume that both mentioned as well as other freshwater shrimps may get in contact with *A. astaci* vectors, particularly with *P. clarkii*, in the pet trade as well as in the wild (Turkmen and Karadal 2012; Uderbayev et al. 2017; Putra et al. 2018).

Methods

Studied decapods and *A. astaci* strains

A. gabonensis is relatively abundant in West Africa, occurring from the Democratic Republic of Congo to Senegal. There are also reports of its presence in South America, however, these are probably erroneous and concern its congener, *A. scabra* (Leach, 1816) (Hobbs and Hart 1982; De Grave and Mantelatto 2013). *A. moluccensis* has a wide distribution ranging from Sri Lanka to Thailand, Malaysia, Indonesia and possibly the Philippines (Chace 1983). Tested individuals of both species were caught in the wild, *A. gabonensis* in Niger, and *A. moluccensis* in Thailand, and subsequently obtained in the Czech Republic from the wholesaler. The Australian yabby, *Cherax destructor* Clark, 1936 originated from an experimental culture kept at the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice (FFPW USB), Vodňany, Czech Republic. The European *A. astacus* was caught with a permit for research purposes (permit no. KUJI 39435/2011 OZP 268/2011/Vac/6) from Pařez pond, Vysočina Region, Czech Republic. The animals were acclimated to the laboratory experimental conditions for a month prior to the beginning of the experiment. The total body length of shrimps (from the tip of the rostrum to the end of the telson) ranged from 44 to 60 mm. *C. destructor* and *A. astacus* individuals had a total length of 42–73 and 53–78 mm, respectively.

The experimental animals were exposed to zoospores of *A. astaci* strain belonging to the genotype group D (Svoboda et al. 2017). The strain originating from infected marbled crayfish *Procambarus virginalis* Lyko, 2017 was obtained from the German aquarium trade (Mrugała et al. 2015) and is kept at the Finnish Food Authority, Kuopio (culture code Evira10823/13). At present, the axenic culture of this *A. astaci* strain is also kept on RGY agar (Alderman 1982) at the Faculty of Science, Charles University, Prague.

Experimental design

The study consists of two subsequent experiments that were conducted in the facilities of the FFPW USB in Vodňany. The infection experiment lasted 120 days between March and July 2016, and was followed after 20 days by a transmission experiment that lasted a further 130 days until December 2016 (Fig. 1).

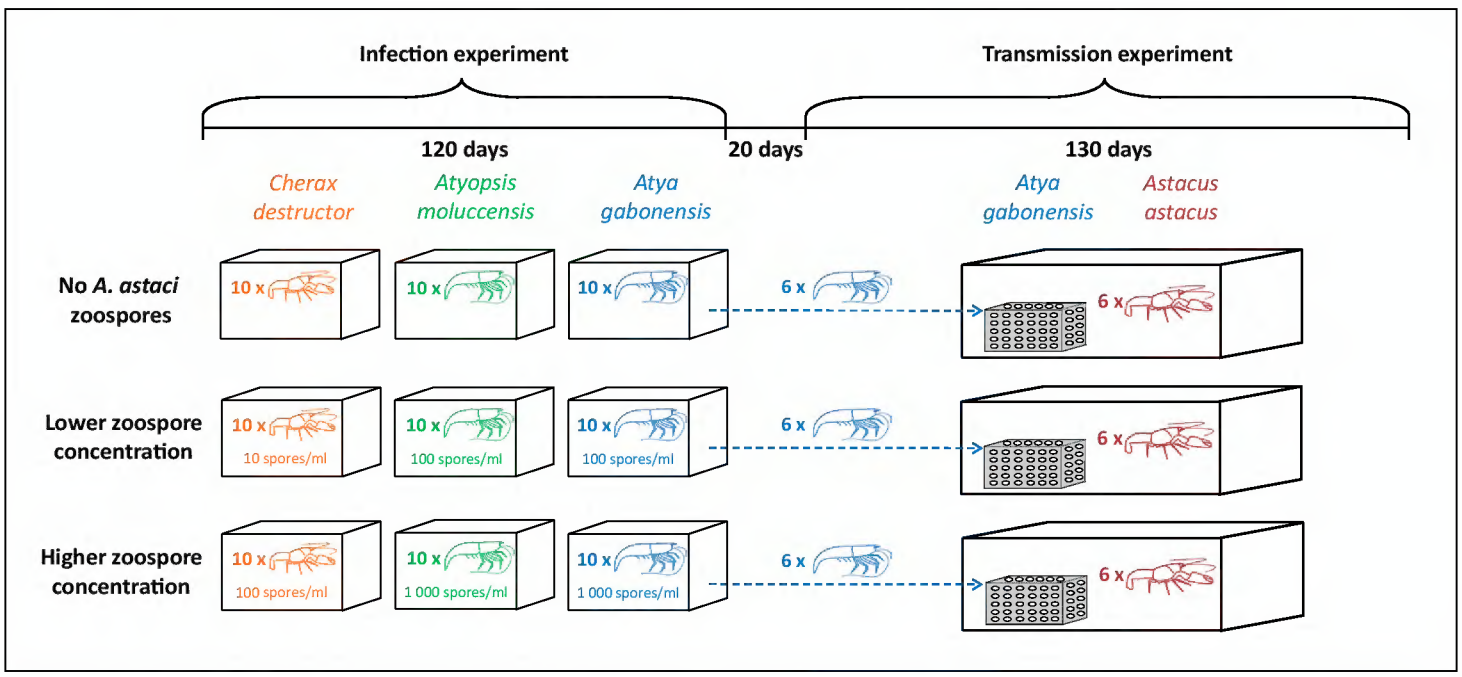

Figure 1. Summary of the experimental design. The study consisted of two subsequent experiments: the infection experiment (120 days long) that was followed after 20 days by a transmission experiment (130 days long). Ten individuals of *C. destructor*, *A. moluccensis* and *A. gabonensis* were used in each of the three treatments: no *A. astaci* zoospores (negative control group) and an addition of one of the two spore doses differing in concentration by an order of magnitude. Six *A. gabonensis* individuals from each treatment were subsequently used in the transmission experiment, and each individual was placed separately with one *A. astacus*. To avoid physical interactions and predation by crayfish, *A. gabonensis* were placed under perforated plastic cages.

Infection experiment

Both shrimp species and *C. destructor* were used in the infection experiment. *C. destructor* served as a sensitive control to evaluate *A. astaci* virulence. This crayfish species was reported to be susceptible to an *A. astaci* strain from the genotype group D (Souty-Grosset et al. 2006) and two other highly virulent *A. astaci* strains belonging to genotype groups B and E (Mrugała et al. 2016). The experimental animals were kept separately in plastic containers (163×118×62 mm) with 1 l of aged tap water under a natural light:dark regime. The weekly water change was preceded by manual cleaning of the containers. The animals were fed daily with 1–3 pellets (Sera Grunugreen, Sera, Germany) depending on their food intake. Water temperature was 19.7±0.4 °C (mean±SD), and concentration of dissolved oxygen was 8.3±0.3 mg·ml^{-1}. To avoid airborne pathogen cross-contamination, no aeration was provided and each container was covered with a plastic lid. The animals were monitored daily; dead shrimps and crayfish as well as exuviae were removed immediately and stored in 96% ethanol.

The *A. astaci* zoospores were produced as described in Mrugała et al. (2016). The experiment was divided into three different treatments: no *A. astaci* zoospores (negative control group) and an addition of two spore doses differing in concentration by an order of magnitude. The spore doses added to containers were 10 spores ml^{-1} and 100 spores ml^{-1} for *C. destructor*, and 100 spores ml^{-1} and 1000 spores ml^{-1} for both shrimp species (Fig. 1). The shrimps were exposed to higher spore concentrations based on their presumed higher resistance to *A. astaci* (Svoboda et al. 2014b). The water volume of 400 ml used during inoculation (due to limited amount of available zoospores) was

increased to 1 l on the next day to ensure suitable conditions for the experimental animals. Ten individuals of each species were used per treatment. Besides 18 *A. gabonensis* individuals used in the subsequent transmission experiment, all surviving animals were euthanised and stored in 96% ethanol after 120 days of the infection trial.

Transmission experiment

Due to a high mortality of *A. moluccensis*, only *A. gabonensis* individuals (six from either treatment, including two infection treatments and negative control group) were used in the transmission experiment. Each potentially infected *A. gabonensis* was kept individually for 20 days in a plastic container. Subsequently, one *A. astacus* individual was placed in each container. To avoid physical interactions and predation by crayfish, *A. gabonensis* were placed under perforated plastic cages. The animals were handled in the same way as during the infection experiment. Water temperature was 18.7±0.3 °C for first 100 days, followed by 23.5±0.2 °C for final 30 days to trigger shedding of the shrimp exoskeleton as zoospore concentrations were observed to increase during crayfish moulting (e.g., Svoboda et al. 2013). Concentration of dissolved oxygen was 8.7±0.6 $mg \cdot ml^{-1}$ and slightly decreased to 6.8±0.8 $mg \cdot ml^{-1}$ during the final 30 days. Upon termination of the experiment, all animals that survived were euthanised and stored in 96% ethanol.

DNA isolation and *A. astaci* detection

All experimental animals were tested for the presence of *A. astaci* DNA in their tissues, presumably indicating infection. Due to a limited number of available animals we did not test any additional individuals for the presence of *A. astaci* infection prior to the beginning of the experiment. The surfaces of all animals were thoroughly rinsed with tap water prior to DNA isolation to remove potentially attached cysts. The total body length of each specimen was measured, and each animal was also examined for any presence of melanised spots on its body, which may indicate a local presence of infection. However, it should be noted that melanisation is a common defence mechanism in crustaceans that can have various causes (Cerenius et al. 2008). As microscopic examination of shrimp tissues for the presence of *A. astaci* hyphae is a non-efficient technique, usually followed by poor results (Svoboda et al. 2014), we omitted this procedure. From each specimen, we dissected soft abdominal cuticle, 2 uropods, 2 legs and if present, any melanised tissues. These mixed-tissue samples were ground in liquid nitrogen, and 50 mg subsamples were subsequently used for DNA extraction with the DNeasy tissue kit (Qiagen) as in Mrugała et al. (2015). The same procedure was used for the DNA extraction from the whole exuviae.

For the detection of *A. astaci* infection, we used the TaqMan minor groove binder real-time PCR assay targeting ITS1 region developed by Vrålstad et al. (2009), with minor modifications of the original protocol introduced later to reduce likelihood of false positive results (as in Svoboda et al. 2014a). The relative levels of infection were

assigned to semi-quantitative agent levels based on the estimated amounts of PCR-forming units (PFU) in the reaction (according to Vrålstad et al. 2009): A0 – no infection, A1 (PFU < 5), A2 (5 ≤ PFU < 50), A3 (50 ≤ PFU < 10^3), A4 (10^3≤ PFU < 10^4), A5 (10^4≤ PFU < 10^5), A6 (10^5≤ PFU < 10^6), A7 (PFU ≥ 10^6).

Statistical analyses

The data analyses were performed in R version 3.4.3 (R Core Team 2017), with the package "survival" (Therneau and Grambsch 2000). Specifically, we evaluated the differences in mortality rates, using the "survdiff" function: 1) between *C. destructor* exposed to the two different zoospore doses, 2) between *A. moluccensis* exposed to the two different zoospore doses, and 3) among all three *A. moluccensis* treatments including the non-infected control. The significance level was set at 0.05.

Results

Infection experiment

No presence of *A. astaci* DNA was detected in any shrimp or crayfish individual from the negative control groups. All *C. destructor* and *A. gabonensis* used in the control groups survived, whereas eight out of ten control *A. moluccensis* died before the end of the experimental trial.

Infection by *A. astaci* was detected in all *C. destructor* individuals from the two zoospore treatments. The infection reached moderate to very high agent levels in crayfish bodies (Table 1), and was observed to be higher in most crayfish exuviae (Appendix 1). Only two *C. destructor* survived in the low-dose treatment and the mortality of the others mostly occurred 42–87 days post-infection (median: 58th day). In the high-dose treatment, all crayfish died between 24 and 104 days post-infection (median: 66th day). No statistical difference was found between these two treatments (χ^2 = 2.2, df = 1, p = 0.135). The moulting and/or loss of limbs occurred shortly before crayfish death in half of the above-described cases.

A. astaci DNA was detected in bodies or exuviae of all *A. moluccensis* and the majority of *A. gabonensis* exposed to *A. astaci* zoospores. The detected *A. astaci* agent levels in the zoospore treatments ranged from very low to low (Table 1), and tended to be higher in the exuviae of moulted individuals (Appendix 1). Furthermore, presence of *A. astaci* infection was no longer confirmed in most *A. moluccensis* bodies after moulting (except of two individuals), indicating the loss of *A. astaci* infection through shedding of exuviae (Appendix 1). Contrasting mortality rates were observed between the two shrimp species. All *A. gabonensis* survived until the end of the experiment, while *A. moluccensis* suffered high mortality. In contrast to the infected crayfish, shrimps did not lose limbs prior to death, and moulting was associated only with two deaths of *A. moluccensis* in the low-dose treatment.

Table 1. Results of the qPCR analyses of crayfish and shrimp bodies after the experimental infection. N: number of individuals of each species exposed to zoospores. Semi-quantitative agent levels based on the estimated amounts of PCR-forming units (PFU) in the reaction (according to Vrålstad et al. 2009) are provided: A2 ($5 \leq$ PFU < 50), A3 ($50 \leq$ PFU $< 10^3$), A4 ($10^3 \leq$ PFU $< 10^4$), A5 ($10^4 \leq$ PFU $< 10^5$), A6 ($10^5 \leq$ PFU $< 10^6$), A7 (PFU $\geq 10^6$).

Species	Zoospore dose (spores ml^{-1})	N	Agent level in infected animals (died during exp./ survived exp. infection)						Survival rate (%)
			A2	A3	A4	A5	A6	A7	
Cherax destructor	10	10			3/2		5/0		20
	100	10				5/0	5/0		0
Atya gabonensis	100	4*	0/1						100
	1000	4*	0/1						100
Atyopsis moluccensis	100	10	4/1	2/0					10
	1000	10	1/0	3/0					40

* six *A. gabonensis* were used in the transmission experiment. Their infection status is provided in Table 2.

Among *A. moluccensis*, the mortality occurred 14–101 days post-infection (median: 23rd day, one surviving individual) in the low-dose treatment and 15–86 days post-infection (median: 32nd day, four surviving individuals) in the high-dose treatment. No statistical difference was found between these two treatments ($\chi^2 = 0.6$, df = 1, $p = 0.439$). The high mortality, however, was also observed among the control individuals, not differing significantly from either infected *A. moluccensis* group ($\chi^2 = 0.6$, df = 2, $p = 0.737$). Specifically, eight control *A. moluccensis* died 14–115 days after the experiment started (median: 29th day, two surviving individuals).

Transmission experiment

Similarly to the infection experiment, no *A. astaci* DNA was detected in the control *A. astacus* and *A. gabonensis*. The shrimp individuals were exposed to *A. astaci* spores prior to the transmission experiment and their infection status was confirmed only after its termination. In the low-dose treatment, *A. astaci* DNA was detected in two shrimps and in exuviae of another individual, whereas in the high-dose treatment *A. astaci* DNA was detected in all shrimps, either in their bodies or exuviae (Table 2). The increased temperature during the last 30 days of the experiment induced moulting in the majority of shrimp individuals. Four, three and four shrimps moulted in the control, low-dose and high-dose treatments, respectively. The *A. astaci* infection reached very low levels in tested shrimp bodies, and very low to moderate levels in their exuviae (Table 2, Appendix 1).

Four individuals of *A. gabonensis* were partially eaten by the *A. astacus*, which managed to reach shrimps despite the attempted physical separation. Three *A. astacus* died during the experiment, however, no *A. astaci* DNA was detected in their tissues. However, a very low agent level was detected in one *A. astacus* individual at the end of the treatment. The cohabiting *A. gabonensis* individual moulted after the increase in temperature and trace amounts of *A. astaci* DNA were detected in its exuviae (Table 2).

Table 2. Results of the qPCR analyses of *Atya gabonensis* and *Astacus astacus* from the transmission experiment. Semi-quantitative agent levels based on the estimated amounts of PCR-forming units (PFU) in the reaction (according to Vrålstad et al. 2009) are provided: A0 – no infection, A1 (PFU < 5), A2 (5 ≤ PFU < 50), A3 (50 ≤ PFU < 10^3), A4 (10^3≤ PFU < 10^4), A5 (10^4≤ PFU < 10^5), A6 (10^5≤ PFU < 10^6), A7 (PFU ≥ 10^6).

Treatment of *A. gabonensis* (spore ml^{-1})	Aquarium number	Agent level			
		Bodies		Exuviae	
		A. gabonensis	*A. astacus*	*A. gabonensis*	*A. astacus*
100	1	A0	A0	A0	
	2	A2	A0		A0
	3	A0	A2	A1	
	4	A2	A0		
	5	A0	A0	A0	
	6	A0	A0	A2	
1000	1	A2	A0	A0	
	2	A0	A0	A2	
	3	A0	A0	A2	A0
	4	A0	A0	A3	
	5	A0	A0	A4	
	6	A2	A0	A0	A0

Discussion

It was assumed for decades that crayfish are the only hosts of *A. astaci*. Unfortunately, recent studies provided evidence that *A. astaci* does not only grow within the tissues of freshwater-inhabiting crabs (Svoboda et al. 2014a; Tilmans et al. 2014; Putra et al. 2018) but can also be successfully transmitted from crabs to European crayfish species (Schrimpf et al. 2014). Whether freshwater shrimps may similarly act as resistant *A. astaci* carriers remained, however, unresolved (Svoboda et al. 2014b). Our study corroborated the results of Svoboda et al. (2014b) by demonstrating an elevated resistance to *A. astaci* infection in two other shrimp species. Furthermore, the outcomes of the exploratory transmission experiment suggest that shrimp individuals previously exposed to *A. astaci* zoospores might, under circumstances favourable for release of zoospores, transmit *A. astaci* to susceptible crayfish hosts.

The elevated resistance of North American crayfish hosts to *A. astaci* has been attributed to the rapid response of their immune system that efficiently limits parasite growth in their cuticles. This defence mechanism is an outcome of long co-evolutionary history between *A. astaci* and its North American crayfish hosts (Unestam and Weiss 1970; Cerenius et al. 2003). It is unlikely that freshwater-inhabiting crabs and shrimps are similarly well-equipped against *A. astaci*; nonetheless; both groups seem resistant to the crayfish plague pathogen. Our results indicate that the tested shrimp species may be capable of resisting *A. astaci* infection; however, their response to the experimental treatments and holding conditions differed. The African *A. gabonensis* were unaffected by either exposure to *A. astaci* or maintenance in small containers, while the Asian *A. moluccensis* suffered extensive mortalities, likely caused by its considerably lower food intake that led to depletion of energy reserves. Because the death rate of *A. moluccensis* control individuals was comparable with individuals exposed to zoospores, it is reasonable to assume that *A. astaci* infection was not the main cause of their mortality.

The progress and success of *A. astaci* infection may be also influenced by the frequent moulting of its hosts, especially those exhibiting increased resistance (Vrålstad et al. 2011; Svoboda et al. 2014b; Mrugała et al. 2016). In our experiment, the exuviae shed by both shrimp species were considerably more infected than the shrimp bodies. Indeed, the parasite penetrates host bodies through the exoskeleton cuticle (Oidtmann 2012), and higher concentration of *A. astaci* DNA is thus expected in this part of the host's body before rather than after moulting. Nevertheless, *A. astaci* DNA was still detectable in shrimp bodies or exuviae after one or even two moulting events indicating that either *A. astaci* had penetrated the soft cuticle or re-colonized the hosts after moulting by zoospores released during that process. As shedding of old cuticle would remove any attached spores, *A. astaci* DNA should be only detectable from the growing *A. astaci* hyphae in these individuals. The *A. astaci* spores were observed to survive for at least 14 days under experimental conditions at 15 °C (CEFAS 2000); the temperature that was close to the one provided during our experiments. Furthermore, Svoboda et al. (2014b) were still able to detect *A. astaci* DNA on filters after seven weeks at 20 °C; however, it remained questionable whether any active zoospores were still present or the assay only picked non-viable cells or environmental DNA. Although the presence of active *A. astaci* zoospores of viable cysts persisting from the original inoculation cannot be entirely excluded in our experiment, it seems unlikely considering the substantial duration of both experimental trials, weekly cleaning of the boxes, water exchange during the experiments, and subsequent rinsing of shrimp bodies prior to DNA extraction. Finally, the detection of *A. astaci* DNA in moulted individuals from the transmission experiment after more than 8 months since the zoospore exposure highlights that the pathogen must have been able to penetrate and grow in shrimp tissues.

The growth of *A. astaci* in host bodies and the subsequent production of motile zoospores is a prerequisite for its successful transmission to the next host. The horizontal transmission of *A. astaci* between different crayfish species has been widely documented in the experimental settings, aquarium facilities as well as from the wild (e.g., Vey et al. 1983; Diéguez-Uribeondo and Söderhäll 1993; Mrugała et al. 2015; James et al. 2017). Our findings highlight that shrimps might also have a potential to transmit *A. astaci* to susceptible crayfish species. Although only one *A. astacus* individual tested positive for *A. astaci* presence, we might have been unsuccessful in detecting this parasite in lowly infected individuals. This was apparently the case in the cohabiting shrimp individual that likely harboured such low level of infection that it only demonstrated trace DNA amounts in the exuviae. Schrimpf et al. (2014) also failed to detect *A. astaci* in tissues of four crabs even though *A. astacus* cohabiting with them got infected. The patchy distribution of the parasite in the host tissues may decrease detection success, especially in resistant hosts (Vrålstad et al. 2009; Schrimpf et al. 2014). Future research on the conditions of *A. astaci* sporulation in alternative hosts should be coupled with observations of the infection's development in their tissues. This would provide important information about the mechanisms behind *A. astaci* horizontal transmission between different decapod hosts and the likelihood of alternative hosts actually releasing zoospores in sufficient numbers for a successful spread of the disease.

A recent experimental study confirmed an elevated resistance to *A. astaci* also in the Australian *C. destructor* (Mrugała et al. 2016). Although all *C. destructor* died after exposure to zoospores of two highly virulent *A. astaci* strains, their mortality was substantially delayed compared to the mortality of *A. astacus*, indicating the potential of *C. destructor* to slow down the development of *A. astaci* infection (Mrugała et al. 2016). Here, we tested susceptibility of *C. destructor* to another highly virulent *A. astaci* strain (genotype group D) isolated from ornamental *P. virginalis*. It is worth noting that these two, as well as many other ornamental crayfish species, get into contact in the aquarium trade (Mrugała et al. 2015). The observed mortality among infected *C. destructor* in our experiment was high. Nevertheless, two individuals survived as long as 120 days after exposure to the lower spore dose, although reaching moderate (A4) infection levels. Therefore, our findings confirm that *C. destructor* should be considered a moderately resistant crayfish species with a potential to transmit *A. astaci* to other decapods.

Among all commercially used crayfish species, the red swamp crayfish *P. clarkii* (the typical host of *A. astaci* genotype group D) has become the most cosmopolitan crayfish introduced to almost all continents, except Australia and Antarctica, thanks to its intensive use for aquaculture, stocking purposes and as an ornamental species (Loureiro et al. 2015). Infected *P. clarkii* individuals in regions as distant as South America and South-East Asia were reported to be responsible for *A. astaci* transmission to native decapods: endemic crayfish *C. japonicus* in Japan (Martín-Torrijos et al. 2018), native shrimp *M. lanchesteri* and crab *P. convexa* in Indonesia (Putra et al. 2018), and possibly also to native crayfish species *Parastacus deffosus* Faxon, 1898 and *P. pilimanus* (von Martens, 1869) in Brazil (Peiró et al. 2016). The potential of freshwater shrimps to act, similarly to *P. clarkii*, as resistant *A. astaci* carriers is alarming and should be further explored. These shrimps or other freshwater decapods may facilitate *A. astaci* spread to susceptible crayfish in aquarium and aquaculture facilities as well as in the wild; particularly in South America, South-East Asia and other regions rich in native crayfish fauna.

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Appendix 1

Table A1. *Aphanomyces astaci* infection levels in bodies and exuviae of animals that moulted during both experiments. Semi-quantitative agent levels based on the estimated amounts of PCR-forming units (PFU) in the reaction (according to Vrålstad et al. 2009) are provided: A0 – no infection, A1 (PFU < 5), A2 (5 ≤ PFU < 50), A3 (50 ≤ PFU < 10^3), A4 (10^3 ≤ PFU < 10^4), A5 (10^4 ≤ PFU < 10^5), A6 (10^5 ≤ PFU < 10^6), A7 (PFU ≥ 10^6).

Species	Concentration (spore ml^{-1})	Animal	Agent level in animal body	Agent level in exuviae		
				Moulting 1	Moulting 2	Moulting 3
Cherax destructor	10	1	A4	A6		
		2	A6	A7		
		3	A6	A7		
		4	A4	A6		
		5	A6	A6		
		6	A4	A6		
		7	A4	A6		
		8	A4	A4	A6	
	100*	1	A6	A4		
Atyopsis moluccensis	100	1	A0	A2		
		2	A2	A3		
		3	A0	A2		
		4	A0	A3		
		5	A2	A3		
	1000	1	A0	A3	A0	A3
		2	A0	A4	A0	
		3	A0	A3		
		4	A0	A2		
		5	A0	A4	A0	
		6	A0	A3	A0	
Atya gabonensis	100	1^{T*}	A0	A1		
		2^{T}	A0	A2		
	1000	1^{T*}	A0	A2		
		2^{T*}	A0	A2		
		3^{T*}	A0	A3		
		4^{T*}	A0	A4		
		5	A0	A4		
		6	A0	A2		
		7	A0	A4		

^{T} moulting in the transmission experiment

^{T*} moulting in the transmission experiment after temperature increase

* only one *C. destructor* from the high-dose treatment moulted during the experiment due to a high moulting rate during acclimation period prior to the addition of the zoospores